

TITLE OF THE INVENTION

METHOD OF DETERMINING ANALYTICAL NUCLEOTIDE SEQUENCE
USED IN NUCLEIC ACID DETECTION

CROSS-REFERENCE TO RELATED APPLICATIONS

5 This is a Continuation Application of PCT
Application No. PCT/JP00/00610, filed February 4, 2000,
which was not published under PCT Article 21(2) in
English.

10 This application is based upon and claims the
benefit of priority from the prior Japanese Patent
Application No. 11-027736, filed February 4, 1999,
the entire contents of which are incorporated herein by
reference.

BACKGROUND OF THE INVENTION

15 1. Field of the Invention

 The present invention relates to a method of
determining a nucleotide sequence of an analytical
oligo nucleic acid for analytical use (referred to as
an analytical oligo nucleic acid, hereinafter), which
20 is used to detect the nucleotide sequence of a specific
nucleic acid. More specifically, the present invention
relates to a method of quickly and efficiently
determining the nucleotide sequence of the analytical
oligo nucleic acid which can detect a predetermined
25 partial nucleotide sequence of an extremely long
nucleotide sequence of a nucleic acid. Therefore, the
method has effective utility in the determination of

nucleotide sequences for nucleic acid analysis.

2. Description of the Related Art

In the method of the present invention, the nucleotide sequence of a desired analytical oligo nucleic acid can be simply and efficiently determined by combining a plurality of calculation processes which can be carried out by an apparatus having a limited calculation capacity.

In general, in a double-stranded chain in which nucleic acids form a hybrid, when a pair of bases facing each other do not satisfy the base pairing according to Watson-Crick base pair (adenine-thymine or uracil, or cytosine-guanine), it is said that a mis-hybridization occurs or a mismatch takes place. If a mismatch occurs, the thermal stability of the hybrid generally decreases. Thus, the mismatch can be avoided by raising the hybridization temperature. However, if a hybrid is formed with the analytical oligo nucleic acid of 30-nucleotide long and the formed hybrid has a single mismatch at the 3' terminal or the 5' terminal, its thermal stability is almost the same as that of the completely-matched hybrid. In this case, even if the hybridization temperature is raised, it is difficult to distinguish a mismatched hybrid from a matched hybrid. Therefore, when such a mis-hybridization takes place, false detection will result.

To overcome the mismatch, all possible structures

assumed by hybrids formed of the nucleotide sequence of
a designed analytical oligo nucleic acid and a target
nucleic acid to be detected are required to be deduced
through calculation, and further, it is required to
5 demonstrate that the nucleotide sequence of the
designed analytical oligo nucleic acid would not form a
mismatch as mentioned above. However, it takes a long
time to deduce all the possible structures through
calculation. Therefore, in a conventional designing
10 for the nucleotide sequence of the analytical oligo
nucleic acid, it has not been evaluated in advance how
strong the designed candidate sequence specifically
hybridizes with a target site. As a result, even if
the structure is deduced by spending a lot of time on
15 calculation, good results are not obtained. Therefore,
the candidate sequences obtained after elaborate work
are obliged to be discarded in many cases.

Detecting the nucleotide sequence of a specific
nucleic acid present in a biological sample is not only
20 important in analyzing a protein which is expressed and
functioning in a specific organ at a molecular level,
and thereby studying expression control of a protein
during information transmission in the nervous, brain
or immune system, but also an important technique for
25 gene diagnosis used for detecting mutant genes in
genetic diseases, diagnosing cancers, and detecting
virus-associated genes. Particularly, the gene

diagnosis is used for final diagnosis where no mistakes are allowed. Furthermore, in the field called "molecular computing", when performing the operation of combinatorial problem using DNAs, the nucleotide
5 sequence of a nucleic acid has to be accurately detected in order to verify what solution has been obtained.

In gene analysis for analyzing the presence or absence of the gene and mutation on the basis of
10 qualitative and quantitative detection of various types of nucleic acid molecules, the most important is to determine a nucleotide sequence of the oligo nucleic acid for use in analysis (hereinafter referred to as "analytical sequence") which forms a double strand
15 specifically with only a predetermined site of a target nucleic acid. In a nucleic acid hybridization reaction, mis-hybridization is likely to occur in the case where the employed analytical sequence is analogous to a complementary sequence at any site
20 except for the target site of the gene to be detected. Therefore, the analytical sequence tends to be designed so as to have a length as long as possible in order to improve the specificity to the target sequence. However, the longer the probe sequence, the more stable
25 the secondary structure of the analytical oligo nucleic acid itself. As a result, hybridization efficiency of the probe with the target nucleic acid significantly

decreases and a hybridization temperature increases.
As a consequence, the hybridization reaction will be complicated.

5 Furthermore, much experience, and trial and error
are required to select the analytical sequence.
In addition, a conventional calculation method for
determining a probe sequence having a stringent
specificity requires enormously amount of time for
calculation. Under these circumstances, it has been
10 increasingly and strongly demanded that the analytical
sequence be easily designed based only upon calculation
without depending upon experience and without
performing numerous preliminary experiments.

BRIEF SUMMARY OF THE INVENTION

15 A first object of the present invention is to
provide a method of determining a nucleotide sequence
of an analytical oligo nucleic acid, namely, an
analytical sequence having a high specificity and
capable of always performing a highly efficient
20 hybridization reaction.

A second object of the present invention is to
provide a method of rapidly determining an analytical
sequence having a high specificity.

25 A third object of the present invention is to
rapidly and economically provide a desired analytical
sequence by using a simple apparatus such as a personal
computer.

The aforementioned objects are attained by a method of determining a nucleotide sequence of an analytical oligo nucleic acid for use in analysis of the nucleic acid, comprising:

5 listing all unit nucleotide sequences present on a target nucleic acid to be analyzed and having a predetermined length which is shorter than the analytical oligo nucleic acid to be designed; and

10 extracting a nucleotide sequence containing a sequence occurring at a low frequency on the target nucleic acid from the candidate sequences of the analytical oligo nucleic acids, as an analytical sequence suitable for analysis for the nucleotide sequence of the target nucleic acid, on the basis of
15 occurrence frequency of the individual unit sequences listed.

It is preferable that the extraction step be performed by successively applying a plurality of different processing procedures.

20 It is preferable that the extraction step further comprises a step of selecting candidate sequences on the basis of chemical properties of individual candidate sequences. In this case, the sequence can be effectively determined since selection is made on the
25 basis whether the probe sequence is suitable or not for hybridization reaction. In particular, if the thermal stability of a molecular structure is employed as the

chemical property for the selection criteria, selection can be made depending upon suitability for the hybridization reaction. As the chemical property for the selection criteria, either thermal stability of a double strand formed of the candidate sequence or the stability of a secondary structure of the candidate sequence, or both are preferable.

Furthermore, in the present invention, there is provided a method of determining a nucleotide sequence for use in detecting a nucleic acid sequence, comprising:

a first calculation step of calculating the occurrence frequency of each of n unit sequences (hereinafter referred to as " n unit sequence") formed of n number of nucleotides (n is an integer of 2 or more) occurring on a nucleotide sequence of a known nucleic acid, on the basis of 4^n types which correspond to all of the n unit sequences;

a first extraction step of extracting, from p unit sequences formed of p number of nucleotides (p is larger than n by m ; and m is an integer of 1 or more), any p unit sequences present on the target nucleic acid to be analyzed;

a second calculation step of calculating a occurrence frequency index of each of the p unit sequences present on the target nucleic acid to be analyzed, on the basis of the occurrence frequency of

the n unit sequences obtained in the first calculating step; and

5 a second extraction step of extracting, as the probe sequence, a p unit sequence having a lower occurrence frequency index obtained in the second calculation step.

10 In the first calculation step, it is preferable that n be any one of 5, 6, and 7. In this case, all types of n sequences which are the bases for obtaining frequencies are 1024 for n=5, 4096 for n=6, 16384 for n=7. These figures are acceptable for the first calculation step, which enables the calculation to be performed at a practical processing speed. The length of each of the p unit sequences in the first extraction
15 step can be set at any value sufficiently to synthesize a nucleic acid probe, for example, within p=10-50.

20 Furthermore, it is preferable that, in at least the second extraction step, the p unit sequence for the analytical sequence be selected from a plurality of p unit sequences having a low occurrence frequency, taking chemical conditions into consideration. As the chemical conditions, the stability of a molecular structure is preferably used, and a T_m value and/or a stability of an intramolecular secondary structure
25 are more preferably used. When both the T_m value and the stability of a secondary structure are used, it is preferable that the selection step is performed by

first selecting a plurality of p unit sequences having a T_m value of a predetermined range, and then further selecting the p unit sequences having an unstable secondary structure from the p unit sequences selected on the basis of the T_m value.

Furthermore, the amount of calculation performed in the sequence determination method described above is relatively low. Therefore, if all steps are sequentially performed by a computer, it is possible to determine an analytical sequence easily and at a low cost. In this case, ultra speed calculation as that of supercomputer is not required, and thus, an advantage is obtained that a generally-used personal computer can be used.

The stability of the secondary structure may be used as an indication for determining whether or not the nucleic acid molecule makes an intramolecular hybrid within the nucleic acid molecule itself. If the nucleic acid probe forms a stable secondary structure within the molecule itself, it is difficult to form a desired hybrid between the probe and a target nucleic acid. The stable secondary structure used herein includes a loop formed of a nucleic acid and partial hybridization of the probe nucleic acid molecules with each other. The nucleic acid forming no stable secondary structure efficiently binds to the sequence of a target nucleic acid when the target nucleotide

sequence is analyzed.

The probe sequence obtained in the present invention is used not only to detect the nucleic acid sequence of a gene but also to detect a nucleic acid having an artificially synthesized sequence and a partial sequence. More specifically, the probe sequence can be used to detect a specific nucleotide sequence of the artificially synthesized nucleic acid, to detect a specific cDNA included in a cDNA library, or to detect a sequence of an exon portion in a genomic sequence of eukaryote. Furthermore, it is possible to detect not only a nucleotide sequence in a genomic DNA of a living organism and a nucleotide sequence of a messenger RNA, but also copies thereof and partial sequences thereof. Moreover, the method of the present invention can be used to design a probe sequence for various enzyme reactions using a hybridization reaction of a nucleic acid, such as the primer used in PCR (Polymerase Chain Reaction).

Additional objects and advantages of the invention will be set forth in the description which follows, and in part will be obvious from the description, or may be learned by practice of the invention. The objects and advantages of the invention may be realized and obtained by means of the instrumentalities and combinations particularly pointed out hereinafter.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWING

The accompanying drawings, which are incorporated in and constitute a part of the specification, illustrate presently embodiments of the invention, and together with the general description given above and the detailed description of the embodiments given below, serve to explain the principles of the invention.

FIG. 1 is a flow chart schematically showing a procedure according to the method of the present invention;

FIG. 2 is a schematic view showing how to set a tuple as a unit sequence;

FIG. 3 is a schematic view showing how to set a primary candidate for an analytical sequence;

FIG. 4 is a graph showing a distribution of occurrence frequency of the unit sequence on a nucleic acid;

FIG. 5 is a graph showing a distribution of T_m values of candidates for the analytical oligo nucleic acid, calculated on the basis of the nucleotide sequences; and

FIG. 6 is a schematic view showing possible shapes of the analytical oligo nucleic acids under hybridization conditions.

DETAILED DESCRIPTION OF THE INVENTION

The method of the present invention will be explained with reference to the accompanying drawings.

However, the present invention will not be limited by the following explanation.

FIG. 1 is a flow chart schematically showing an embodiment of the method according to the present invention. More specifically, FIG. 1 shows the steps of designing a nucleotide sequence of a probe nucleic acid serving as an analytical oligo nucleic acid for use in gene identification. In the embodiment, a probe nucleic acid is designed for detecting a specific ORF (Open Reading Frame) on the genome of an Escherichia coli (E. coli). Prokaryote such as E. coli does not have an Exon/Intron structure although eukaryote has. Therefore, most of ORFs of the prokaryote correspond the nucleotide sequence of a gene. To be more specific, detecting a specific ORF means detecting a specific gene. In the embodiment, there is provided a high-speed algorithm in which a calculation amount increases in proportion to the length of a genome.

First, the nucleotide sequence of the entire genome of E. coli is scanned to completely list all unit sequences each constituted of 7 nucleotides (hereinafter, referred to as "7 tuple") present in the genome. For example, as shown in FIG. 2, the nucleotide sequence consisting of first to seventh nucleotides from an appropriate terminal (sequence) of Genome 1 is determined as a first tuple 2. Thereafter, the frame consisting of the first 7-tuple 2 is shifted

by one nucleotide on the genome to obtain the second 7-tuple, the third 7-tuple, the fourth 7-tuple and so on. If the procedure is sequentially repeated, all 7 tuples can be completely listed. Subsequently, all the 7 tuples are classified into types based on the nucleotide sequences thereof, and then, all kinds of 7 tuples are checked as to how many number of each of 7-tuples are present on the genome.

Next, the all kinds of 7 tuples classified based on individual nucleotide sequences are checked for the occurrence frequency thereof. If the frequency is regarded to be an existence rate, the total number of 7 tuples existing on Genome 1 has to be employed as a denominator. However, the sum of rates of existence is not necessary to reach 100% in the present invention. It is sufficient if relative frequencies of different 7 tuples occurring on the genome are obtained. For this reason, it is practical to employ the number of mathematically possible 7-tuple combinations as the denominator, for convenience sake. To explain more specifically, since a nucleotide sequence of a gene is constituted of four types of nucleotide-bases (adenine, thymine, guanine, and cytosine), the types of nucleotide sequences possibly constituting the 7 tuples will be $4^7 (=16384)$ in theory. In more general, the number of types will be 4^n when an n -tuple unit is used.

Using the figure of 4^7 (= 16384) as the denominator, the occurrence frequency of each 7-tuple present on the entire genome is obtained (First calculation step). In this case, comparison can be readily made by using a graph as shown in FIG. 4 in which individual tuples are plotted on the lateral axis and their frequencies are plotted on the vertical axis. The calculation and graph-drawing mentioned above can be readily made by using a commercially-available computer. Note that the data as to frequencies of individual 7-tuples are stored in a memory.

As shown in FIG. 3, all the candidate sequences possibly used as an analytical sequence are completely listed, wherein the analytical sequence consists of nucleotides the number of which is larger than that of the 7-tuple by at least one nucleotide, preferably 10-50 nucleotides (e.g., 30 nucleotides), and is present on the ORF to be detected. With respect to each candidate sequence of 30-nucleotides, an index of the occurrence frequency is calculated on the basis of the occurrence frequency of the 7-tuple obtained in the first calculation step, as described below.

There are twenty four 7-tuples in the 30 nucleotides. Assuming that the twenty-four 7-tuples are numbered 1 to 24 sequentially from the side of the 5' terminal of the 30 nucleotides, and that the frequencies corresponding to the twenty-four 7 tuples

calculated above are represented by $p_1, p_2 \dots p_{24}$.
In this case, the occurrence frequency index of the
30-nucleotide analytical sequence can be calculated by
multiplying the frequencies of the twenty-four 7 tuples
with each other, as represented by $p_1 \times p_2 \times \dots$
5 p_{24} . The occurrence frequency index indicates how
specifically a candidate sequence hybridizes with the
ORF to be detected. The lower the value of the index,
the higher the specificity. The occurrence frequency
10 index is calculated with respect to all 30-nucleotide
candidate sequences present on the target ORF.
The candidate sequences are selected based on
an appropriate threshold value of the index.
The candidate sequences selected in this calculation
15 step are referred to as "low occurrence frequency
candidate sequence group". Note that the calculation
and graph-drawing can be readily performed by
a commercially available computer. Data of the
occurrence frequency of individual 30 nucleotide
20 partial sequences are stored in a memory.

A group of low occurrence frequency candidate
sequences extracted in the above is evaluated for other
conditions other than the occurrence frequency, that
is, physicochemical conditions, thereby selecting
25 a desired probe sequence. The probe sequence is not
determined by the occurrence frequency alone. This is
because the probe having a nucleotide sequence specific

to a target sequence does not always form the hybrid efficiently. It is therefore preferable that each of low occurrence frequency candidate sequences be checked for thermal stability, as shown in FIGS. 5 and 6.

5 First of all, T_m values are plotted on a graph shown in FIG. 5. Then, the low occurrence frequency candidate sequences within a predetermined range of T_m values are selected. The T_m values are calculated based on, for example, a SantaLucia parameter (John SantaLucia, Jr.,
10 Hatim T. Allawi, and P. Ananda Seneviratne "Improved nearest-neighbor parameters for predicting DNA duplex stability." Biochemistry 35, 3555-3562). The reason why the sequences having T_m values of the predetermined range are chosen is that the plurality of analytical
15 nucleotide sequences which have specificities to the corresponding ORFs and satisfy the T_m requirement can hybridize with the ORFs simultaneously under the same temperature. The remaining low occurrence frequency candidate sequences which are not eliminated in the
20 aforementioned selection step, are regarded as more potential candidate sequences, so that they are checked for the stability of a secondary structure formed within a molecule itself.

For example, as shown in FIG. 6, analytical
25 nucleic acids are immobilized on a solid-phase support 4 with an appropriate linker molecule 5 interposed between them. When the construct is placed in a

5 solution mixture containing a reactive substance such
as a test sample, the stability of the molecular
structure can be discussed as follows. A candidate
probe 6 forms a loop called "auto-hybrid" in the
molecule. A candidate sequence 7 partially forms an
intermolecular hybridization with another analytical
sequence immobilized on the support 4. Since the
secondary structures of these probes are stable, it may
be difficult or impossible for them to form a desired
10 hybrid with the target nucleic acid. Therefore, these
probes capable of forming stable secondary structures
are eliminated. As a result, a partial sequence 8
which is capable of readily hybridizing with a target
under hybridization conditions, is selected as the most
15 potential candidate sequence. The stability of the
secondary structure can be calculated based upon the
nucleotide sequence by use of an appropriate analytical
software.

20 Finally, the most potential candidate sequences
which are selected on the basis of the occurrence
frequency and physicochemical conditions are further
checked for their availability as the analytical
sequence for identifying the ORF. The availability is
checked by using a full length of the genome of
25 Escherichia coli. More specifically, whether or not
the selected analytical nucleotide sequence binds
complementarily to an only one specific position of

the genome is checked. The analysis for binding specificity is performed by using a computer. For example, a local binding map is first formed by a dynamic programming method and then the nucleotide sequences of the entire genome of Escherichia coli are compared to the map. In this manner, it can be verified that it is not feasible for the sequence to cause a mis-hybridization. The verification step may be also performed by calculating Hamming distance between the most potential candidates sequences and the nucleotide sequence of the entire genome of Escherichia coli. The nucleotide sequence passed the verification step is determined as the analytical nucleotide sequence.

When detection is performed based on hybridization using the analytical sequence determined in the above, a marker probe is prepared by binding a detectable marker substance to the oligo nucleic acid having the analytical sequence. The hybridization reaction can be performed in a predetermined manner in which the marker probe and a test sample are mixed together, and then, the hybridized marker substance is selectively measured. If a fluorescent substance such as FITC (Fluorescein Isothiocyanate) is employed as the marker substance, the detection can be readily made by an appropriate fluorescent detector. Further, by using a data processing means, quantitative or qualitative

analysis can be automatically performed. In this case,
it is possible to determine the presence or absence of
the target nucleic acid molecule or a reaction amount
on the basis of qualitative or quantitative measurement
5 data (numerical value or image). The results of the
gene analysis can be obtained by printing the result
on a paper sheet as a report or displaying the result
on a screen.

The hybridization reaction of nucleic acids
10 is used not only for detecting a gene but also in
a nucleic acid amplification reaction such as PCR, and
furthermore, used in an identification reaction such as
LCR (Ligase Chain Reaction).

The oligo nucleic acid having the analytical
15 sequence designed according to the method of the
present invention can be used as a primer in the PCR or
as a probe in the LCR. Furthermore, plural types of
analytical sequences may be appropriately applied to a
single genome depending upon a principal of detection.
20 The analytical oligo nucleic acid may be immobilized on
a solid phase support such as microparticles, chip
substrate, column, filter, test paper, well.

The present invention is not limited to aforemen-
tioned embodiments and may be modified in various ways
25 on the basis of the gist of the present invention.
For example, all steps explained in FIG. 1 may be
automatically performed. In this case, it is

5 sufficient that only the finally determined probe
sequence is displayed or output. Depending upon the
user's wish, it may be possible to omit the screen
display or print-out of the graph for an occurrence
frequency of the tuple, and data and graph with respect
to a binding site of the analytical sequence on
a target nucleic acid to be detected, and the T_m
value and a secondary structure of the analytical
sequence. Alternatively, individual calculation steps,
10 calculation with respect to T_m value and the stability
of the secondary structure, and conversion of the
results into numerals or a graph may be performed by
a computer, whereas evaluation including a final
selection may be performed by a user on the basis of
15 the numerical data or graph displayed on the screen
display. In this case, the data extracted or selected
herein may be input by using an input means such as
a keyboard or mouse. In addition, the data obtained
through various computation or calculations are not
20 always necessary to be stored in a memory etc. In the
automatic operation, various calculation data and the
results of extraction and determination may be
exchanged with various institutes such as hospitals,
universities, examination centers by mutually
25 transmitting them on an on-line network connecting
these institute and a host computer.

Evaluation steps of the T_m value and the stability

of the secondary structure may be performed in inverse order or at the same time. When the most preferable analytical nucleotide sequence is selected from the extracted low occurrence frequency candidate sequences under the condition in which a first preference is given to the physicochemical conditions, the analytical nucleotide sequence may be selected so as to include a tuple whose occurrence frequency is not the lowermost one.

In the method of the present invention can be applied to not only a genomic nucleotide sequence, but also expressed messenger RNA and cDNA (a copy of RNA), and further, artificially synthesized DNA may be used as a target. More specifically, the method of the present invention is used to design an analytical nucleotide sequence directed to a specific nucleotide sequence of any one of the aforementioned targets.

Example

A PCR experiment was performed using primers designed by the method of the present invention for amplification of a mouse genes by PCR method, as described below.

The nucleotide sequences of all genes of a mouse (balb/c) were not elucidated. Therefore, the nucleotide sequence of a mouse (balb/c) registered in GenBank as of September 5, 1999 was used by assuming it as the entire nucleotide sequence of a mouse.

Primers shown below were prepared for amplifying the DNA of a mouse.

group	Gen Bank note	Name of Gene
1	138444	TGTP/Mg21
2	m15525	Lamini B1
3	m18194	Fibronectin
4	m35725	Cu/Zn-SOD
5	m37030	Diff6

5 The sequences of the primer obtained through calculation, calculated lengths of the amplified products, and Tm values are shown in a table below. Note that the primer sequence is written from the 5' end. It took two hours to perform calculation for obtaining the primer sequences under the following
10 conditions. If the same calculation is performed without using the tuple method of the present invention, it takes 11.5 hours or more.

Computer used herein

15 CPU: Pentium III 500 MHz
RAM: 384 Mbyte
OS: Linux
Compiler: c++

Number of primer pair	Calculated T _m (°C)	Length of amplification product (bp)	Primer Sequence on the forward chain	Primer Sequence on the reverse chain
1	54	122	TGGGACCATATATATCTGAGCCCCCGAGT (SEQ ID No. 1)	CCCATCGGGACTAGGCTAAAAATCGTGCCC (SEQ ID No. 2)
2	62	151	TAAGGACATAAGTGAGAAAGTTGCGGTTTA (SEQ ID No. 3)	GGTGGTTAAAAACATTAAATAGATGATGGG (SEQ ID No. 4)
3	54	267	GTTCTTATGGTTTGGTCTGGGATCAATAGG (SEQ ID No. 5)	CTGGGAAAAAATTGATAAAATAACAAACAGGT (SEQ ID No. 6)
4	62	86	TGATTGGGATTGCGCAGTAAACATTCCCTG (SEQ ID No. 7)	CAGATTACAGTTTAATGGTTTGAGGGTAGC (SEQ ID No. 8)
5	62	276	GAGGGTAGGCCCTGCCCTTGCACTTAAACCA (SEQ ID No. 9)	GAATAGGAAAAACCCCAATTGGAACGCGGGAA (SEQ ID No. 10)

PCR condition

Reaction solution: Constituent per 50 μ L

Template: manufactured by Clontech.

0.4 μ g of Genomic DNA

extracted from a mouse

(balb/c) liver

Enzyme manufactured by TaKaRa.

ExTaq 5 units

dNTP (mixture of dATP, dCTP, dGTP, dTTP):

2.5 nmol for each

Buffer for ExTaq: manufactured by TaKaRa,

Mg²⁺ concentration 2 mM)

Primer: 20 pmol for each

Temperature Cycle conditions for PCR

(1) 95°C 30 seconds

(2) 65°C 60 seconds

(3) 72°C 60 seconds

To improve stringency, the temperatures were set higher than required. Steps (2) and (3) were repeated

30 cycles.

Electrophoresis conditions

gel: manufactured by FMC

Nusieve GTG agarose 4% TAE buffer

Voltage and time: 100V, 30 minutes

Results

Amplified products by the PCR reaction were obtained with expected lengths.

As described in the above, according to the method of the present invention, it is possible to determine an analytical sequence which can always accurately hybridize with a target nucleotide sequence.

5 Furthermore, according to the present invention, it is possible to quickly determine the analytical sequence. Furthermore, according to the present invention, the analytical sequence is determined step by step by combining relatively small-amounts of calculations
10 without requiring a large calculation capacity. Therefore, a large-size computer is not required. Hence, determination of the analytical sequence can be simply performed by using an economically favorable computer for general use.

15 Additional advantages and modifications will readily occur to those skilled in the art. Therefore, the invention in its broader aspects is not limited to the specific details and representative embodiments shown and described herein. Accordingly, various
20 modifications may be made without departing from the spirit or scope of the general inventive concept as defined by the appended claims and their equivalents.

- 26 -
SEQUENCE LISTING

<110> METHOD OF DETERMINING ANALYTICAL NUCLEOTIDE SEQUENCE USED IN
NUCLEIC ACID DETECTION

<120> OLYMPUS OPTICAL CO., LTD

<130> 99S1124P

<140> JP/1999-027736

<141> 1999-02-4

<160> 10

<170> PatentIn Ver. 2.0

<210> 1

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 1

tgggaccata tatatctgag ccccccgagt

30

<210> 2

<211> 30

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<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 2

cccatcggga ctaggctaaa aatcgtgccc

30

<210> 3

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 3

taaggacata agtgagaaag ttgcggttta

30

<210> 4

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 4

094947 073004

ggtggttaaa aacattaaat agatgatggg

30

<210> 5

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 5

gttcattatgg ttggtctgg gatcaatagg

30

<210> 6

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 6

ctgggaaaaa ttgataaata acaaacaggt

30

<210> 7

<211> 30

<212> DNA

<213> Artificial Sequence

<223> Description of Artificial Sequence: primer

tgattgggat tgcgcagtaa acattccctg

30

<211> 30

⟨213⟩ Artificial Sequence

<223> Description of Artificial Sequence: primer

cagattacag tttaatgggtt tgagggtagc

30

<211> 30

⟨213⟩ Artificial Sequence

<223> Description of Artificial Sequence: primer

gagggtaggc ctgcccttgc acttaaacca

30

$\langle 210 \rangle$ 10

<212> DNA

 $\langle 220 \rangle$

<223> Description of Artificial Sequence: primer

 $\langle 400 \rangle$ 10

30